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**(54) Title:** NOVEL COMPOSITIONS AND METHODS FOR INHIBITING LIGHT-INDUCED INACTIVATION OF BIOLOGICAL REAGENTS**(57) Abstract**

Disclosed is a fluorescent conjugate comprising a biological reagent, a fluorescent molecule, and a means for impeding phototoxic degradation of the biological reagent. The impeding means can be a cross-linking substance having a long molecular distance, whereby the cross-linking substance links the fluorescent molecule and the biological reagent; a quencher of singlet oxygen; a quencher of a free radical; or a combination thereof. Also disclosed is a solution comprising a fluorescent conjugate of a biological reagent and a fluorescent molecule together with an oxygen depleting system.

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DESCRIPTIONNovel Compositions and Methods For Inhibiting Light-Induced  
Inactivation of Biological Reagents5 Field of the Invention

This invention relates to the stabilization of fluorescent conjugates, in particular to stabilization of biological reagents, such as proteins, peptides, ligand analogues and nucleic acids, conjugated to a fluorescent molecule or to a  
10 fluorescent particle.

Background Art

In order to determine the presence or concentration of target ligands or analytes in a fluid, immunoassays utilize  
15 binding proteins, such as antibodies, to specifically bind to target ligands or analytes. Commonly, a signal is generated from the immunoassay to provide a detectable or sensible result. A class of useful signal generators are fluorescent molecules because of their high specific activity.

20 Fluorescent molecules have been utilized in immunoassays as signal generators in several ways. For example, enzymes can catalyze the production of fluorescent dyes from a substrate, such that the amount of fluorescent dye generated is related to the presence or amount of analyte or target ligand in a sample;  
25 such enzymes can be conjugated to antibodies or ligand analogues. Fluorescent dyes can also be conjugated to antibodies or ligand analogues, and the amount of fluorescent signal is measured as a result of the assay process to define the analyte presence or amount.

30 An objective with immunoassays is to make them more sensitive, so that a lower concentration of analyte can be measured. To increase sensitivity, one can conjugate a biological reagent to a fluorescent particle. As used herein,

"fluorescent particles" can be formed of a synthetic material such as latex, can comprise a natural material such as liposomes, or combinations of natural and synthetic materials. In the preparation of the fluorescent particles, the fluorescent dyes or enzymes are generally imbibed into and/or adsorbed onto the particles, using mixed organic and aqueous solutions. The fluorescent dyes or enzymes incorporated into particles are usually water insoluble, whereas the dyes or enzymes conjugated directly to biological reagents are generally water soluble.

One can conjugate, for example, a biological reagent to a fluorescent particle that contains many fluorescent molecules or enzymes capable of converting a substrate into a fluorescent product. In the case of conjugating an antibody to a fluorescent particle, the analyte that is bound by the antibody conjugated to the fluorescent particle containing many fluorescent molecules is thus associated with a larger signal as compared with binding an antibody that is conjugated with a single fluorescent molecule or enzyme.

Examples of immunoassays that incorporate fluorescent dyes or enzymes are numerous and some are described in U.S. Patents 4,283,382; 4,351,760; 4,420,568; 4,868,132; 4,876,190; 5,075,215; and, E.P.O. Application Number PCT/US87/03226.

Immunoassays are characterized as either competitive or non-competitive. Competitive immunoassays generally operate by a competition between a ligand analogue signal generator complex and a ligand (i.e., the analyte) for binding to a limited amount of antibody. Competitive immunoassays can also function by a competition between a protein or an antibody signal generator conjugate and an analyte which is a protein or an antibody, respectively, for binding to a limited amount of ligand. Non-competitive immunoassays generally function by having two different antibodies bind the analyte at different epitopes, one antibody conjugated to the signal generator and the other antibody in solution or bound to a solid phase in

order to facilitate separation of unbound signal generator from the bound signal generator.

The antibody concentration in an immunoassay generally dictates the kinetics of the immunoassay, as well as the fraction of analyte bound to the antibody. In general, to accelerate the binding of an analyte to an antibody so that the reaction achieves a desired reaction rate or equilibrium in a desired time, the highest antibody concentration that is feasible is used in the assay; this is particularly true for non-competitive immunoassays. Thus, a high antibody concentration is often employed in fluorescent assays. Generally a high antibody concentration is also associated with a high fluorescent dye concentration, since each make up the antibody:particle conjugate or the antibody:dye conjugate.

Thus, for immunoassays, fluorescent particles are often used to obtain a more sensitive measure of analyte concentration. The quantity of a fluorescent molecule that needs to be associated with a complex of an analyte and a fluorescent conjugate is generally determined based on the sensitivity requirements of the assay. Thus, for sensitive assays, the amount of fluorescent dye or enzyme associated with the analyte in the assay process, is maximized. In such a case, the local concentration of fluorescent molecules is very high in the immediate vicinity of the biological reagent because they are in close proximity to each other.

Nucleic acid assays also utilize fluorescent signal generators. Signal generators associated with nucleic acids are used in assays to measure nucleic acids complementary with the nucleic acid associated with a signal generator. In order to optimize the hybridization kinetics with the target nucleic acid and also to achieve the most sensitive assay, these nucleic acids are also at a high local concentration in the vicinity of fluorescent molecules. To create a nucleic acid conjugate, a signal development element is conjugated to a

nucleic acid sequence. The signal development element can be a fluorescent particle or a fluorescent molecule.

Previously, however, a problem was encountered with fluorescent conjugates for use in assays. Often, fluorescent conjugates were not stable. The reactivity of such conjugates was found to decay over time, such that the function or properties of a biological reagent in a fluorescent conjugate would change with time. For example, the binding affinity of an antibody linked to a fluorescent signal would often decrease with time. The mechanism(s) for this degradation of the properties of biological reagents in fluorescent conjugates was not known. It was presumed, however, that the degradation occurred consequent to the release or uncoupling of the biological reagent from the fluorescent molecule.

#### Disclosure of the Invention

For the first time in the art, the mechanisms leading to the degradation of biological reagents in fluorescent conjugates has been identified. Accordingly, compositions and methods are provided to modulate this degradation, both in fluorescent conjugates and in the reagents used to synthesize the fluorescent conjugates. The effectiveness of fluorescent conjugates for use in assays is enhanced pursuant to the present invention.

Thus, disclosed is a fluorescent conjugate comprising a biological reagent, a fluorescent molecule, and a means for impeding phototoxic degradation of the biological reagent. The impeding means can comprise a cross-linking substance having a long molecular distance, whereby the cross-linking substance links the fluorescent molecule and the biological reagent; a protein; a quencher of singlet oxygen; a quencher of a free radical; a system for depleting oxygen; or a combination thereof.

The conjugate can comprise that the fluorescent molecule is directly linked to the biological reagent. The conjugate

can further comprise a particle which comprises a fluorescent molecule; the particle can comprise a natural material or a synthetic material. For example, the particle can comprise a natural material which is alumina, silica, or, a liposome. The particle can further comprise an oxygen depleting system.

Disclosed is a solution comprising a fluorescent conjugate and an oxygen depleting system. For an embodiment of the fluorescent conjugate to be placed in a solution, the solution can comprise an means for impeding phototoxic degradation. The impeding means of the solution can comprise an antioxidant, a protein, an oxygen depleting system, or a combination thereof. The fluorescent conjugate of the solution can comprise a biological reagent, a fluorescent molecule, and a means for impeding phototoxic degradation of the biological reagent. The impeding means of the conjugate in the solution can comprise: a system to deplete oxygen; a cross-linking substance having a long molecular distance, whereby the cross-linking substance links the fluorescent molecule and the biological reagent; an antioxidant; a protein; or, a combination thereof.

Also disclosed is a fluorescent conjugate prepared by a process comprising steps of: providing a biological reagent; providing a fluorescent molecule; providing a heterofunctional linking reagent; chemically reacting the biological reagent, the fluorescent molecule, and the heterofunctional linking reagent, whereby the biological reagent becomes linked to the fluorescent molecule; and; associating an oxygen depleting system, a protein, an antioxidant, or a combination thereof, with the biological reagent or the fluorescent molecule prior to the reacting step; or, associating an oxygen depleting system, a protein, an antioxidant, or a combination thereof, with the linked biological reagent and fluorescent molecule following the reacting step. The heterofunctional linking reagent can be: a heterofunctional polyethylene glycol (PEG) derivative, a long chain form of succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), a long chain.

form of succinimidyl 3-(2-pyridyldithio)propionate (SPDP), or, a heterofunctional peptide derivative.

Disclosed is a method of use of an antioxidant, a protein, an oxygen depleting system, or a cross linking substance to  
5 impair the phototoxic degradation of a biological reagent in a fluorescent conjugate comprising the biological reagent and a fluorescent molecule, or to impair the phototoxic degradation of the material used to prepare a composition comprising a fluorescent molecule, the method occurring by combining an  
10 antioxidant, a protein, an oxygen depleting system, or a cross linking substance with the fluorescent conjugate, or with the material used to prepare a composition comprising a fluorescent molecule.

Also disclosed is a method to facilitate preparation of a  
15 stable fluorescent composition, where the composition comprises a fluorescent molecule which absorbs light and provides a fluorescent result upon exposure to light of a defined spectrum. The method comprises: providing an environment comprising diminished amounts of light from the defined  
20 spectrum, relative to a white light environment; providing the fluorescent molecule, or a material to prepare the composition; placing the fluorescent molecule or the material to prepare the composition into the provided environment. An embodiment of the method is disclosed which comprises: providing the  
25 fluorescent molecule; providing the material to prepare the composition; placing the fluorescent molecule into the provided environment; placing the material to prepare the composition into the provided environment; and, combining the fluorescent molecule and the material to prepare the composition; whereby  
30 phototoxic degradation of the material to prepare the composition is diminished upon completion of the method. When this embodiment of the method is performed, the combining step can occur before the step of placing the fluorescent molecule into the provided environment and before the step of placing

the material to prepare the composition into the provided environment.

### Definitions

5       Fluorescent molecule - As used herein, a fluorescent molecule is one that either emits light during the transition from an excited electronic state to a lower energy state, or is an enzyme or catalyst that is capable of acting on a substrate to yield a molecule that emits light during the transition from  
10 an excited electronic state to a lower energy state.

Fluorescent dyes - Fluorescent dyes are those that emit light during the transition from an excited electronic state to a lower energy state. The lower energy state is commonly referred to as the ground state. The excited state is attained  
15 by absorption of light at an appropriate wavelength. One pathway for relaxation of the excited state is the emission of light. Fluorescent dyes exhibit singlet-singlet emission of light.

Fluorescent particles - A particle which comprises one or  
20 more fluorescent molecules. A fluorescent particle can be formed of a synthetic material such as latex, can comprise a natural material such as liposomes, or can comprise a combination of natural and synthetic materials.

Ligand - A binding partner to a ligand receptor or to a  
25 complementary nucleic acid sequence.

Ligand analogue - A chemical derivative of a ligand. A ligand analogue may be attached either covalently or noncovalently to another species, for example, to a signal development element. Ligand analogue and ligand may be the  
30 same and both are capable of binding to ligand receptor.

Ligand Receptor - A receptor capable of binding ligand. Typically, a ligand receptor is an antibody or a complementary nucleic acid sequence, but may also be a ligand.

Signal Development Element - The element of the biological  
35 reagent conjugate that produces a detectable signal. The

signal can be directly produced by the signal development element, or produced upon exposure of the signal development to a substrate.

5 Radical - A radical is an atom or a group of atoms that possess an unpaired electron. Radicals are also referred to as free radicals, diradicals and radical pairs.

Biological Reagent - A moiety, e.g., a protein, peptide, ligand analogue, or nucleic acid capable of being associated with a signal development element in a conjugate.

10 Fluorescent conjugates - A fluorescent conjugate comprises a biological reagent, e.g., a protein, peptide, ligand analogue or nucleic acid sequence, in association with a fluorescent molecule, a fluorescent particle, an enzyme, or a generator of fluorescent dyes. The association can be covalent or non-  
15 covalent.

The fluorescent molecules can be conjugated with natural or synthetic particles to form fluorescent particles. For example, a fluorescent molecule can be adsorbed to alumina or silica particles, or imbibed into latex particles or liposomes;  
20 the proteins, peptides, ligand analogues or nucleic acids can be adsorbed or covalently coupled to the particles to make the fluorescent conjugates. Alternatively, fluorescent molecules can be directly coupled to the proteins, peptides, ligand analogues or nucleic acids to form fluorescent conjugates. The  
25 direct coupling can be a covalent bond. The fluorescent molecules utilized for these latter conjugates are generally water soluble having a carboxylic acid, thiol, amine, maleimide, alkyl halide or N-hydroxy succinic ester moiety to facilitate coupling to the proteins, peptides, ligand analogues  
30 or nucleic acids.

Antioxidant - A substance such as a quencher which, when present in a system significantly delays or inhibits oxidation of the substrate.

Reactive species - Examples of reactive species are  
35 singlet oxygen, free radicals and excited triplet states.

Quencher - A substance which either through physical means, such as energy transfer, or through chemical means delays or inhibits the decomposition of another substance.

## 5 Modes For Carrying Out Invention

For the first time in the art, the mechanisms that lead to the degradation of biological reagents in fluorescent conjugates have been identified. It has been found that ambient white light leads to the generation of singlet oxygen or radicals which act on biological reagents. The studies that led to this finding are disclosed, as are compositions and methods to modulate this degradation. Pursuant to the present invention, the effectiveness of fluorescent conjugates for use in assays is enhanced.

15 Although the degradation of biological reagents in fluorescent systems had been noted, the causative mechanism was unknown. Surprisingly, it was found that biological reagents such as antibodies, peptides and ligand analogues, as well as reagents used to synthesize the fluorescent conjugates, are degraded by a mechanism that is induced upon exposure of these materials to routine ambient conditions. The ambient conditions can constitute mere exposure to white light and oxygen.

25 Accordingly, this invention concerns the stabilization of proteins, peptides, ligand analogues and nucleic acids that are comprised in fluorescent conjugates, as well as stabilization of the materials to make fluorescent conjugates.

## Mechanism(s) of Degradation of Biological Reagents

30 The inactivation of antibody binding activity in immunoassays or inactivation of nucleic acid hybridization assays by singlet oxygen or radicals has not been previously described. Previously, only the inactivation of lysozyme and the oxidation of amino acids have been described (Singlet Oxygen, 1985, Vol. IV, p. 91-143, ed. A.A. Frimer, CRC Press,

35

Boca Raton, FL; Int. J. Radiat. Biol. (1986) 50:41-45); however, these descriptions are related to fluorescent dyes that are not conjugated to proteins, peptides, ligand analogues and nucleic acids; unlike the present invention, these dyes and biological reagents were freely soluble in aqueous solutions.

Furthermore, it has been noted that phthalocyanines can also initiate phototoxicity by an electron transfer mechanism (Type I) in which the fluorescent dye, in an excited triplet state, can undergo reactions with neighboring molecules by an electron or hydrogen transfer process. The production of singlet oxygen (Type II) by the excitation of fluorescent dyes has been utilized for photodynamic therapy (Polym. Adv. Technol. (1995) 6:118-130; Int. J. Radiat. Biol. (1995) 67:85-91; Ciba Foundation Symposium (1989) 146:17-26) and in immunoassays, whereby singlet oxygen generation by particles comprised of silicon phthalocyanine has been used to generate a delayed luminescence. The delayed luminescence is proportional to the analyte concentration (Clin. Chem. 42:1518-1526).

The teachings herein show that light (ambient or focused) decreased the effectiveness in binding reactions of antibodies, peptide and ligand analogue fluorescent conjugates. The mechanism for the decrease in effectiveness is believed to be related to the generation of reactive species, such as singlet oxygen or radicals, by the fluorescent molecules. The inactivation of biological reagents in fluorescent conjugates makes them unreliable for use in assays. The inactivation of antibody binding in immunoassays or of nucleic acid hybridization in nucleic acid assays is generally manifest by a decrease in the slope of a dose response curve of the assay.

As disclosed herein, light-mediated inactivation of biological reagents in fluorescent conjugates is very dramatic. It has been found that the degradation of biological reagents in fluorescent conjugates is independent of the fluorescent molecule used in making the fluorescent conjugate. The efficiency of the inactivation is believed to be a result of two

factors: First, the high local concentrations of both the dye and the biological reagent; and, second, the relative proximity of the biological reagent and the fluorescent dye in the fluorescent conjugates.

5       The presumed mode of degradation is type I and/or type II mechanisms of phototoxicity. In the case of type I mechanisms, the triplet sensitizer, that is, the triplet state of a fluorescent dye, reacts with nearby molecules through an electron or hydrogen transfer process. Free radicals are thus  
10   formed; namely the semireduced sensitizer and the semioxidized substrate. The semireduced sensitizer can react with oxygen to form a superoxide radical anion, which can further react with other molecules. The semioxidized sensitizer can become reduced by another molecule to form a semioxidized substrate,  
15   which can further react.

      In the case of type II mechanisms, the triplet sensitizer can react with ground state oxygen to form singlet oxygen which can further react with other molecules, such as biomolecules and reagents to give oxidized products. (See, e.g., Int. J. Radiat. Biol. (1995) 67:85-91).

20       As disclosed herein, biological reagents associated with fluorescent dyes become inactivated or ineffective through the triplet state of the dye. The generation of triplet states of fluorescent dyes can result in the formation of a variety of  
25   radical species, including but not limited to singlet oxygen, superoxide radicals, hydroxyl radicals and organic radicals. These radical species have been known to cause damage to biological cells and proteins. Accordingly, these reactive triplet state species react with the surface of the biological  
30   reagent. Consequently, there will be a decreased effectiveness of an antibody for binding to analyte, or for a peptide or ligand analogue for binding to antibody. The inventive teachings set forth compositions which slow or prevent the light-induced destruction of biological reagents.

Stabilization Of Biological Reagents

This invention relates to the stabilization of biological reagents conjugated to fluorescent particles or conjugated to a fluorescent molecule, as well to the preparation of such  
5 conjugates. The biological reagent can comprise a protein, peptide, ligand analogue or nucleic acid component. When a biological reagent is stabilized in accordance with the invention, phototoxic degradation of the biological reagent in a fluorescent conjugate is impeded.

10 Accordingly, it has now been found that inactivation of biological reagents conjugated to fluorescent molecules or fluorescent particles can be decreased or prevented by use of radical scavengers termed quenchers, oxygen depleting systems in solutions that contain the fluorescent biological reagent  
15 conjugates, chemical linkages in conjugates to minimize the effects of radicals, or combinations thereof.

Another embodiment of this invention comprises a system which consumes dissolved oxygen. For example, a system of this  
20 embodiment comprises catalyse, glucose oxidase, and glucose, as discussed in greater detail herein.

In a preferred embodiment, solutions containing chemically oxidizable compounds that are associated with or conjugated to fluorescent molecules, also contain singlet oxygen and/or free  
25 radical quenchers to minimize or prevent oxidation of the chemical species. Oxidizable compounds include those that are capable of being converted from one oxidation state to a higher one. In general, most oxidations involve a gain of oxygen and/or a loss of hydrogen.

In another preferred embodiment, solutions containing  
30 biological reagents that are associated with or conjugated to fluorescent molecules, also contain singlet oxygen and/or free radical quenchers to minimize or prevent inactivation of the biological reagent. Biological reagents include, peptides and proteins containing oxidizable amino acids or pendant  
35 molecules, and nucleic acids.

In another preferred embodiment, solutions containing antibodies that are associated with or conjugated to fluorescent molecules or particles also contain singlet oxygen and/or free radical quenchers to minimize or prevent the ability of the antibody to bind its antigen. Antibodies include, polyclonal and monoclonal antibodies and binding fragments generated by recombinant protein synthesis or other techniques.

In yet another preferred embodiment, solutions containing nucleic acids that are associated with or conjugated to fluorescent molecules, also contain singlet oxygen and/or free radical quenchers to minimize or prevent the inactivation or slow the rate of hybridization of the nucleic acid to a complementary nucleic acid strand.

Compounds that can be utilized as quenchers of singlet oxygen include, but are not limited to those listed in Table 1. Compounds that can be utilized as quenchers of free radicals include, but are not limited to, those listed in Table 2.

#### Table 1

##### Singlet Oxygen Quenchers

carotenoids: naturally occurring and synthetic tocopherols and tocopheramines: homologues, isomers, derivatives and related compounds

thiols: e.g. glutathione, ergothionine, cysteine, N-acetyl-cysteine, dihydrolipoic acid, thiol-containing proteins and peptides)

amino acids: tryptophan, tyrosine, histidine, methethione, cysteine, and peptides and proteins containing these amino acids

probucol

2,2,6,6-tetra-methyl-piperidine (TEMP)

plamitoyl ascorbic acid

caffeine

squalene

esters of polyunsaturated fatty acids  
flavonoids  
lidocaine  
imidazole and derivatives  
5 phthalocyanines and naphthalocyanines  
p-aminobenzoic acid (PABA)  
curcumin  
spermine  
spermidine  
10 merocyanine 540  
cholesterol  
azide

Table 215 Free Radical QuenchersMetal Complexes

manganese (II) stearate  
manganese (II) acetate  
20 bis-(acetylacetonate)manganese (II)  
N,N'-ethylene-bis-(salicylideneiminato)manganese (II)  
bis-(dimethylglyoximate)-bis-pyridineferrous  
ferric stearate  
tris-(acetylacetonato)ferric  
25 bis-(dimethylglyoximate)ammineiodidecobalt (II)  
bis-(dimethylglyoximate)amminechloridecobalt (II)  
tris-(dimethylglyoximate)cobalt (II)  
bis-(dimethylglyoximate)ammineiodidecobalt (II)  
bis-(dimethylglyoximate)pyridineiodidecobalt (II)  
30 bis-(dimethylglyoximate)pyridinechloridecobalt (II)  
bis-(dimethylglyoximate)-bis-pyridinecobalt (II)  
cobalt (II) stearate  
cobalt (II) chloride  
cobalt (II) acetate  
35 cobalt (II) cyclohexylcarboxylate

- bis-(acetylacetonato)cobalt (II)  
N,N'-ethylene-bis-(salicylideneiminato)cobalt (II)  
porfirine cobalt (II)  
bis-(salicylate) nickel  
5 N,N'-ethylene-bis-(salicylideneiminato)nickel  
N,N'-ethylene-bis-(N-ptoluidinyl)salicylideneiminato)nickel  
copper stearate  
bis-(dimethylglyoximato)copper  
bis-(salicylate)copper  
10 bis-(dimethylglyoximato)copper  
bis-(diphenylglyoximato)copper  
bis-(dimethylglyoximato)ammineiodidecopper  
copper sulfate  
copper acetate  
15 bis-(acetylacetonato)copper  
porfirine copper

#### Phenols

- hydroxybenzenes  
20 benzoquinons  
catechol and derivatives  
cresol isomers (o-, m-, p-)  
chromans  
dihydrobenzofurans  
25 1,2-ethane-2,2'-bis-(4,6-di-tert-butylphenol)  
hydroxyfluorenes  
hydroquinone  
4,4'-methane-bis-(2,6-di-tert-butylphenol)  
dihydroxynaphthalene  
30 naphthol isomers  
pentaerithritol ester of 3,5-di-tert-butyl-4-  
hydroxyphenylpropionic acid  
hydroxyphenantrene isomers  
phenol and derivatives

pentaeritryl tetrakis[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate]  
ethyleneglycol bis-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate  
5 resorcinol  
silane, tetra(ethyloxy-3,5-di-tert-butyl-4-hydroxyphenyl)  
4,4'-sulfide-bis-(3,5-di-tert-butyl-4-dydroxybenzyl)  
2,2'-sulfide-bis-[4-methyl-5-hydroxy-6-(1'-phenyl)ethyl]  
2,2'-disulfide-bis-[4-methyl-6-hydroxy-5-(1'-phenyl)ethyl]  
10 indophenol  
tetrahydroquinoline, 5,7,8-trimethyl-6-hydroxy-N-acetamido  
tetrahydroquinoline, 5,7,8-trimethyl-6-hydroxy-N-ethyl  
tocol, 5,7-dimethyl  
tocopherol isomers ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ )

15

#### Aromatic Amines

carbasol  
mono- and di-naphthylamines  
diphenylamine and derivatives  
20 p-phenylenediamine derivatives  
phenylamine  
phenylnaphthylamine isomers  
4-phenyloxyphenyl-2-naphthylamine  
4-(spirotetrahydrofuran-2') spirocyclohexyl-1,2,3,4-  
25 tetrahydroquinoline  
4-tert-butylphenyl-1-(4-tert-butyl)naphthylamine  
1,2,3,4-tetrahydroquinoline, 2,2,4-trimethyl

25

#### Hydroxylamines And Nitroxyl Radicals

30 9-azabicyclo[3.3.1]nonanehydroxylamine  
8-azabicyclo[3.2.1]octanehydroxylamine  
1,4-diazacycloheptane, 2,2,6,6-tetramethyl-5-oxo  
2,5-dihydroimidazole derivatives  
2,5-dihydropyrrol derivatives  
35 3,4-dihydropyrrol derivatives

1,2-dihydroquinoline derivatives  
hydroxylamine derivatives  
piperidine derivatives  
pyrrolidine derivatives  
5 tetrahydroimidazole derivatives  
tetrahydropyridine derivatives  
tetrahydropyrrol derivatives  
tetrahydroquinoline derivatives

10 Thiophenols

thionaphthol isomers  
thiophenol and derivatives  
phenolsulfides

15 Phosphorous And Sulfur Containing Antioxidants

phosphines  
phosphites  
sulfides  
disulfides

20 sulfoxides  
metalthiocarbamates  
metalthiophosphates

Biological Antioxidants

25 Low Molecular Weight, Water Soluble

ascorbate;  
thiols;  
urate;  
bilirubin and biliverdin;  
30 flavins (e.g., vitamin B2);  
quercetin

Low Molecular Weight, Lipid Soluble

carotenoids (natural and synthetic) and related polyenes;  
vitamin A (i.e. retinol, retinal, retinoic acid) and  
35 related compounds;

tocopherol homologues, isomers, derivatives and related compounds;  
ubiquinol and ubiquinone;  
flavonoids

5     Proteins

thiol containing: albumin; thioredoxin; glutaredoxin  
      redox active: cytochrome c; myoglobin  
      enzymes: superoxide dismutase (SOD), catalase, various peroxidases

10    Drugs

      salicylate; ebselen; synthetic thiols and thiol derivatives; probucol and its derivatives;  
      phenylbutylnitrone; spin traps; nitecapone and its analogs; penicillamine; lazaroids; aminosalicylates;  
15    stobadine; nitroxides; tamoxifen and estrogens;  
      plasmalogens; calcium channel blocking drugs; nitroxides; melatonin

      A further aspect of the invention comprises use of  
20 heterofunctional reagents, such as heterobifunctional reagents, which have long molecular distances. The linkages produced thereby were used in fluorescent conjugates to associate a fluorescent molecule with a biological reagent. Accordingly, the biological reagent was at a maximized distance from the  
25 source of radical production. Maximizing the distance of the biological reagent from the source of the radical producer increased the time that the radical must travel, and therefore increased the decay of the radical before it reached and then damaged the biological reagent. Such cross-linking reagents  
30 include but are not limited to: heterofunctional PEGs comprised of N-hydroxysuccinidyl (NHS) esters of poly (oxyethylene) derivatives containing a maleimide or vinyl sulfone group or similar electrophilic moiety; long chain forms of succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), such as  
35 succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxy-(6-

amido-caproate), or of succinimidyl 3-(2-pyridyldithio) propionate (SPDP), such as succinimidyl-6-[3-(2-pyridyldithio) propionamido] hexanoate; and heterofunctional peptide derivatives.

5 An alternative embodiment of this invention utilized a protein substrate which was juxtaposed between a fluorescent molecule and a biological reagent. The protein substrate functioned to interact with the reactive species, such that the reactive species was quenched before it moved into the sphere  
10 of the biological reagent involved in that assay. This led to increased stability of the assay reagents over time. An example of this embodiment is set forth as Example 7.

Another embodiment of this invention incorporated water insoluble quenchers into a fluorescent particle conjugate. Such  
15 quenchers include but are not limited to quenchers of singlet oxygen, free radicals and excited triplet states; examples of these are given in Tables 1 and 2. A preferred quencher is the carotenoid  $\beta$ -carotene, which can quench singlet oxygen, free radicals and excited triplet states; most other quenchers  
20 listed in Tables 1 and 2 possess the identified quenching activities. The water insoluble quenchers in physical contact with the dye molecules interacted efficiently with the reactive species, because they were closer to the source of production of the reactive species than the biological reagent involved in  
25 the assay. An example of this embodiment is set forth as Example 8.

Another embodiment of this invention comprised a combining water soluble quenchers and emulsions of water insoluble quenchers, including but not limited to detergent micelles and  
30 liposomes, with a solution that contained the fluorescent conjugate or fluorescent particle conjugate. Examples of this embodiment are set forth as Examples 5 and 6.

Another embodiment of this invention comprised a combination of water insoluble quenchers incorporated into a  
35 fluorescent particle conjugated to a biological reagent, in

combination with a system which consumes dissolved oxygen present in the solution that contains the conjugate.

Another embodiment of this invention comprised a combination of water soluble quenchers or emulsions of water insoluble quenchers in combination with a system which consumes dissolved oxygen in a solution that contains a fluorescent conjugate.

Another embodiment of this invention comprises a combination of both water insoluble quenchers incorporated into the fluorescent conjugate particle, water soluble quenchers or emulsions of water insoluble quenchers in the solution that contains the conjugate, together with a system which consumes dissolved oxygen in the solution that contains the fluorescent conjugate.

## Examples

### Example 1

#### Effect of Light Exposure on the Activity of Antibodies Coupled to Fluorescent Particles

Particles coupled with anti-troponin antibodies (this and all other antibody conjugates used in this and the following examples as well as the particular fluorescent molecules involved were disclosed, and were prepared as disclosed, in U.S. Patent Application Nos.: 08/409,298 and 08/409,825, each filed 23 March 1995; and U.S. Patent Application No.: 08/620,597 filed 22 March 1996. U.S. Patent Application Nos.: 08/409,298; 08/409,825; and 08/620,597, including all respective parent applications are fully incorporated by reference herein) were diluted to 0.14% solids in air-saturated storage buffer (hereafter called storage buffer, which had the following composition: STABILCOAT (BSI, Eden Prairie, MN), 13% (vol/vol); sucrose, 23 mg/mL; bovine serum albumin (30% solution, manufacturing grade, Bayer Corp. Kankakee, IL), 13 mg/mL; potassium phosphate, 30 mM; potassium borate, 6 mM; sodium chloride, 90 mM; pH 7.5)) to form an incubation mixture

and then split among two colorless 1.5 mL microcentrifuge tubes, one of which was incubated under room light (light provided by standard white fluorescent tubes), and the other protected from light, both at room temperature.

5        Antibody activity was determined by immunoassay by combining an aliquot of the incubation mixture containing antibody conjugates with an equal volume of troponin assay reagent (this reagent consisted of 6 ng/mL human cardiac troponin I (Biotech International, Inc., Seattle, WA) and 2  
10    µg/mL biotinylated goat anti-troponin I peptide 3 specific antibody (BioPacific, Emeryville, CA), which formed a complimentary pair with the anti-troponin antibodies coupled to the fluorescent particles (i.e., a fluorescent conjugate), in assay reagent buffer consisting of 140 mM sodium chloride, 10  
15    mM potassium phosphate, 40 g/L bovine serum albumin (30% lution, manufacturing grade, Bayer Corp., Kankakee, IL), pH 7.4); the resulting sample was incubated for 15 min and then assayed in an immunoassay device as disclosed in U.S. Patent Application 08/423,582 (filed 18 April 1995).

20        Briefly, the sample was loaded at the top of the diagnostic lane of the device and allowed to flow by capillary action through the diagnostic lane that had avidin-HS bound on one surface in a capture zone. After the sample flowed into the device, assay reagent buffer was loaded at the top of the  
25    diagnostic lane to wash away unbound reagents from the capture zone. The amount of antibody conjugate bound to the capture zone was quantified by scanning the diagnostic lane with a fluorometer consisting of a laser diode excitation source (670 nm) and a silicon photodiode detector measuring fluorescence at  
30    the wavelength maximum of 760nm with the appropriate optical filters and electronics to obtain the fluorescence signal. A unit of antibody activity was defined based on the slope of the dose response curve of the assay. Assay activities in Table 3 are expressed relative to the activity of the sample that had

not been exposed to light (i.e., the dark control), which was set at 100%.

Table 3

time of light exposure	% of dark control
0 hours	100
14 hours	25
24 hours	16

5        These results showed that exposure of the antibody conjugates to light resulted in a time dependent loss of assay signal when compared to a dark control.

#### Example 2

#### 10    Effect of Particle Dye Content on the Light Induced Inactivation of Antibodies Coupled to Fluorescent Particles

Antibody conjugates with both anti-troponin and anti-CKMB antibodies were prepared with latex particles containing fluorescent dye at three different concentration levels. The  
15 three particle preparations were diluted to 0.14% solids in argon-purged storage buffer containing 5 mM sodium ascorbate and the glucose oxidase system (10 mg/mL glucose, 10 µg/mL glucose oxidase (Calbiochem, San Diego) and 10 µg/mL catalase (Calbiochem, San Diego)) and then split among two colorless 1.5  
20 mL microcentrifuge tubes, one of which was incubated under room light, and the other protected from light.

After 24 hours, the incubation mixtures were assayed for activity by immunoassay by combining aliquots of each with an equal volume of troponin/CKMB assay reagent (this reagent  
25 consisted of 20 ng/mL each human cardiac troponin I (Biotech International, Inc., Seattle, WA) and CKMB (Genzyme, San Carlos, CA)), 2 µg/mL biotinylated goat anti-troponin I peptide 3 specific antibody (BioPacific, Emeryville, CA) and 2 µg/mL fluorescein-labeled anti-CKMB antibody (IIL-3, IIL Inc.), which  
30 formed a complimentary pair with the anti-CKMB antibodies

coupled to the fluorescent particles, in buffer containing 140 mM sodium chloride, 10 mM potassium phosphate, 40 g/L bovine serum albumin (30% solution, manufacturing grade, Bayer Corp., Kankakee, IL), pH 7.4), incubating for 2 min, and assaying the incubation mixture as described in Example 1. The devices employed in this example had capture zones comprised of immobilized anti-fluorescein antibody and avidin-HS. In Table 4, assay activities are expressed relative to the activity of the samples that had not been exposed to light (i.e., the dark control), which was set at 100%.

Table 4

relative dye content of particle	% of dark control	
	troponin assay	CKMB assay
1	48	30
3	22	19
9	11	13

These results showed a direct, positive correlation between the dye content of the antibody conjugates and the degree of light dependent inactivation of antibodies coupled to the particle. This was seen in both the troponin and CKMB antibody assays.

### Example 3

#### Effect of Green Light on the Activity of Antibodies Coupled to Fluorescent Particles

An incubation mixture of anti-troponin conjugates was prepared as described in Example 1 and was divided among three colorless 1.5 mL centrifuge tubes, which were incubated respectively under green fluorescent light (Model F40G, General Electric), standard white fluorescent light or in the dark.

After 24 hours, each incubation mixture was sampled and assayed as described in Example 1, the results are set forth in Table 5.

5 Table 5

ambient light source	assay activity (Units)
dark (control)	20
white fluorescent tube	4
green fluorescent tube	26

These results showed that light emitted by green fluorescent tubes did not exert the same deleterious effect on antibody activity as seen with full spectrum white light. The  
10 excitation wavelength maximum of the fluorescent dye in the antibody conjugate is 670 nm, which corresponds to the red region of the visible spectrum. The emission spectrum of the green fluorescent light contains much less red light than that of the white fluorescent light; thus, production of  
15 fluorescence was impeded during preparation of the fluorescent conjugates.

#### Example 4

##### Effect of Dissolved Oxygen Deprivation on the Light Induced 20 Inactivation of Antibodies Coupled to Fluorescent Particles

Particles coupled with anti-troponin I antibody were diluted to 0.14% solids in air-saturated storage buffer, in argon-purged storage buffer and in argon-purged storage buffer prepared with 100 mM sodium ascorbate, and were incubated in  
25 colorless 1.5 mL microcentrifuge tubes under room light. Samples were withdrawn from the incubation mixtures and were assayed as described in Example 1. The antibody activity of the conjugates in air-saturated storage buffer was 74, 19 and 12 Units after 0, 14 and 24 hours of exposure to light,

respectively. In Table 6, antibody activity at each time point is expressed relative to the activity of the conjugates kept in air-saturated storage buffer.

5 Table 6

time of light exposure	storage buffer	relative activity
14 hours	air-saturated	1.0
	argon-purged	2.5
	100 mM ascorbate, argon-purged	2.5
24 hours	air-saturated	1.0
	argon-purged	2.9
	100 mM ascorbate, argon-purged	4.4

These results showed that decreasing the level of dissolved oxygen in the incubation mixtures, either by purging the mixture with an inert gas or by including a autoxidizable compound in the mixture, protected the antibody conjugate against light induced inactivation. An advantage of including an antioxidant, such as ascorbate, is particularly evidenced at the longer time point, where ascorbate afforded a greater degree of protection than simply purging the mixture with argon.

#### Example 5

##### Effect of Antioxidants on the Light Induced Inactivation of Antibodies Coupled to Fluorescent Particles

20 Particles coupled with anti-troponin I antibody were diluted to 0.14% solids in air-saturated storage buffer and in argon-purged storage buffer prepared with 20 mM ascorbate and the glucose oxidase system as described in Example 2. Each incubation mixture was divided among two colorless 1.5 mL microcentrifuge tubes, one of which was incubated under room light, and the other protected from light. After 17 hours the

mixtures were assayed for activity by immunoassay by combining aliquots of each with an equal volume of troponin assay reagent, incubating 2 min, and assaying as described in Example 1. The results are set forth in Table 7.

5

Table 7

antioxidants	incubation condition	assay activity (Units)
none	in dark	41
	under room light	6
ascorbate, glucose oxidase system	in dark	36
	under room light	35

These results showed that the addition of an antioxidant and an oxygen consumption system to the storage buffer greatly enhanced the stability of the antibody conjugate to light exposure; here it can be seen that the activity of conjugates incubated in the absence of antioxidants decreased by 85%, whereas conjugates incubated in the presence of an antioxidant and the oxygen consuming system retained their activity.

Example 6Conditions for Stabilizing Antibody Conjugates

Particles coupled with anti-troponin I antibody were diluted to 0.14% solids in argon-purged storage buffer prepared with final concentrations of 50 mM sodium ascorbate, 20 mM Trolox (Aldrich Chemical Co., Milwaukee, WI), 10 mM sodium azide, and 10 mg/mL glucose. The incubation mixture was contained in two clear, colorless glass vials. Glucose oxidase was added to the mixtures and the vials were argon purged. The vials were then sealed with a septum lined crimp cap. One of the vials was wrapped in foil to exclude light, while the other

was left exposed to light. Both vials were placed in a 3-neck round bottom flask, to which a nitrogen gas line, an exhaust line leading to a bubble chamber, and a stopper were attached. The flask was kept under room light and a continuous flow of nitrogen for 3.8 days. Antibody activity was determined as described in Example 1. Results are set forth in Table 8.

Table 8

incubation condition	assay activity (Units)
in dark	25
under room light	25

These results demonstrated that minimizing or eliminating oxygen from antibody conjugates can prevent the light induced activation.

In an additional experiment it was found that samples which were prepared with the glucose oxidase system but without the antioxidants ascorbate, Trolox and azide, and kept under an atmosphere of nitrogen, retained full activity during exposure to light for 7.8 days.

Example 7

Effect of an Intervening Protein Coat on the Light Induced Inactivation of Antibody Conjugates

Particles coupled with anti-troponin I antibody were prepared from latex particles that had been adsorbed with bovine serum albumin prior to antibody coupling, and from latex beads that had not received the bovine serum albumin. The bovine serum albumin was adsorbed to fluorescent particles at 5 mg/mL protein and 0.5% solids. The unbound protein was separated using gel filtration chromatography. The antibody was coupled to the protein coat with SMCC and SPDP (Pierce Chemical Co., Rockford, IL) using standard protein coupling procedures.

Mixtures at 0.14% solids of both of these particles were prepared by diluting them into air-saturated storage buffer and then split among two colorless 1.5 mL microcentrifuge tubes. A tube from each set was incubated under room light and in the dark. After 17 hours samples were withdrawn from each tube and assayed as described in Example 1. Results are set forth in Table 9.

Table 9

protein coat	storage condition	% of dark control
none	in dark (control)	2.9
	under room light	2.9
albumin	in dark (control)	100
	under room light	17

These results showed that particles prepared with an intervening layer of protein were more resistant to light induced antibody inactivation than particles lacking a protein layer. Other protein coats, which were comprised of fibrinogen, cytochrome C and thyroglobulin, also reduced the light induced inactivation of the antibody conjugates (data not shown).

#### Example 8

##### Affect of Water Insoluble Antioxidants Incorporated into the Particle on the Light Induced Inactivation of Antibodies Coupled to Fluorescent Particles

Particles coupled with anti-troponin I antibody were prepared from latex particles that contained the carotenoid lycopene (Sigma Chemical Co., St. Louis, MO) in addition to the fluorescent dye, a separate set of coupled particles was

prepared from latex beads that contained the fluorescent dye but no lycopene. Each of the particle preparations was diluted to 0.14% solids in air-saturated storage buffer and then split among two colorless 1.5 mL microcentrifuge tubes. One tube from each set was incubated under room light and the other was shielded from light.

After 22 hours samples were withdrawn from each tube and assayed as described in Example 1. Assay activities are expressed relative to the activity of the sample that had not been exposed to light. The results are set forth in Table 10.

Table 10

antioxidant	incubation condition	% of dark control
le	in dark (control)	100
lycopene	in dark (control)	100
	under room light	84

These results showed that incorporating an antioxidant such as lycopene into the fluorescent particle decreased the degree of light induced inactivation of the antibody coupled to the particle. Particles prepared with other carotenoids (e.g. lutein,  $\beta$ -carotene, bixin, crocetin and physalene) and antioxidants (e.g. nickel complexes, stilbene and BHA) were also tested and found to be more resistant to light induced inactivation than control particles without these protectors (data not shown).

Example 9

Effect of Antioxidants on the Light Induced Inactivation of Antibodies Coupled to Different Fluorescent Particles

Particles coupled with anti-CKMB antibody were prepared from fluorescent latexes obtained commercially (Molecular Probes, Inc., catalog numbers L7189, L7201 and L7204) and from a different standard fluorescent latex prepared for this and the other experiments herein. Mixtures at 0.14% solids of these particles were prepared by diluting them either into air-saturated storage buffer or into argon-purged storage buffer containing 5 mM sodium ascorbate, 10 mM Trolox, 10 mM sodium azide, 10 mg/mL glucose and 10 µg/mL each catalase and glucose oxidase. Each mixture was split among two clear, colorless glass vials and the vials were sealed with septum lined crimp caps. Vials containing the antioxidant mixtures were evacuated with argon prior to the addition of the glucose oxidase and capping. All vials were placed in a 3-neck round bottom flask, to which a nitrogen gas line, an exhaust line leading to a bubble chamber, and a stopper were attached. The flask was evacuated with nitrogen, sealed and placed under room light. After a 4 day incubation, samples were withdrawn from the vials and assayed for antibody activity immunoassay.

Immunoassays were conducted by combining the samples with an equal volume of CKMB assay reagent (this reagent consisted of 50 ng/mL CKMB (Genzyme, San Carlos, CA) and 2 µg/mL of a biotinylated anti-CKMB antibody, which formed a complimentary pair with the antibodies coupled to the fluorescent particles, in buffer containing 140 mM sodium chloride, 10 mM potassium phosphate, 40 g/L bovine serum (30% solution, manufacturing grade, Bayer Corp., Kankakee, IL, pH 7.4), incubating the reaction mixture for 15 min, and then analyzing it by device assay as described in Examples 1 and 2.

Assay signals were determined using a fluorescence spectrometer (model LS50B, Perkin Elmer, Norwalk, CT) and are expressed as relative fluorescence. Results are set forth in Table 11.

Example 11Use of Heterobifunctional Reagents to Increase the Diffusion Distance for Reactive Species in Conjugates

Heterobifunctional reagents which have long molecular distances were used to prepare fluorescent conjugates. The linkages produced thereby were used to associate a fluorescent molecule with a biological reagent. Such cross-linking reagents include but are not limited to: heterofunctional PEGs comprised of N-hydroxysuccinidyl (NHS) esters of poly (oxyethylene) derivatives containing a maleimide or vinyl sulfone group or similar electrophilic moiety; a heterofunctional crosslinking reagent such as a sulfhydryl and amino reactive heterobifunctional crosslinking reagent; and, heterofunctional peptide derivatives. In preferred embodiments, the sulfhydryl and amino reactive heterobifunctional crosslinking reagent can comprise long chain forms of SMCC or SPDP; e.g., succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxy-(6-amido-caproate) and succinimidyl-6-[3-(2-pyridyldithio)propionamido] hexanoate, respectively. It being understood that other linking agents known in the art can be used in accordance with the invention disclosed and claimed herein.

Accordingly, the biological reagent in a conjugate was at a maximized distance from the source of radical production at the signal moiety. Maximizing the distance of the biological reagent from the source of the radical production increased the time that the radical must travel, and therefore increased the decay of the radical before it reached and then degraded or damaged the biological reagent. Preferred embodiments of the invention comprise conjugates comprising long molecular distances between biological reagent and fluorescent signal moieties, in combination with other inventive embodiments to stabilize the fluorescent conjugates such as an oxygen depleting system, a protein, an antioxidant, or a combination thereof.

Claims

1. A fluorescent conjugate comprising a biological reagent, a fluorescent molecule, and a means for impeding phototoxic degradation of the biological reagent.

2. The conjugate of claim 1 wherein the impeding means comprises a cross-linking substance having a long molecular distance, whereby the cross-linking substance links the fluorescent molecule and the biological reagent.

3. The conjugate of claim 1 wherein the impeding means comprises a protein.

4. The conjugate of claim 1 wherein the impeding means comprises an antioxidant.

5. The conjugate of claim 4 wherein the impeding means comprises an antioxidant which is a quencher of a free radical or a quencher of singlet oxygen.

6. The conjugate of claim 1 wherein the impeding means comprises an oxygen depleting system.

7. The conjugate of claim 1 wherein the impeding means comprises: a cross-linking substance having a long molecular distance, whereby the cross-linking substance links the fluorescent molecule and the biological reagent; a protein; an antioxidant; an oxygen depleting system; or, a combination thereof.

8. A solution comprising the fluorescent conjugate of claim 1.

9. The solution of claim 8 wherein the solution comprises a means for impeding phototoxic degradation of

biological reagent, where the phototoxic degradation means of the solution comprises a protein, an antioxidant, an oxygen depleting system, or a combination thereof.

5        10. The conjugate of claim 1 wherein the fluorescent molecule is directly linked to the biological reagent.

11. The conjugate of claim 1 further comprising a particle which comprises the fluorescent molecule.

10

12. The conjugate of claim 11 wherein the particle comprises a natural material or a synthetic material.

13. The conjugate of claim 12 wherein the particle  
15 comprises a natural material which is alumina, silica, or, a liposome.

14. A solution comprising a fluorescent conjugate and a  
solution-based means for impeding phototoxic degradation of a  
20 biological reagent.

15. The solution of claim 14 wherein the fluorescent  
conjugate comprises a biological reagent, a fluorescent  
molecule, and a means for impeding phototoxic degradation of a  
25 reagent.

16. The solution of claim 14 wherein the impeding means  
of the conjugate comprises: an oxygen depleting system; a  
cross-linking substance having a long molecular distance,  
30 whereby the cross-linking substance links the fluorescent  
molecule and the biological reagent; a protein; an antioxidant;  
or, a combination thereof.

17. The solution of claim 14 wherein the solution-based  
35 means for impeding phototoxic degradation of a biological

reagent comprises: an oxygen depleting system; a protein; an antioxidant; or, a combination thereof.

18. A fluorescent conjugate prepared by a process  
5 comprising steps of:

providing a biological reagent;

providing a fluorescent molecule;

providing a heterofunctional linking reagent;

10 chemically reacting the biological reagent, the  
fluorescent molecule, and the heterofunctional linking reagent,  
whereby the biological reagent becomes linked to the  
fluorescent molecule; and;

15 associating an oxygen depleting system, a protein, an  
antioxidant, or a combination thereof, with the biological  
reagent or the fluorescent molecule prior to the reacting step;  
or, associating an oxygen depleting system, a protein, an  
antioxidant, or a combination thereof, with the linked  
biological reagent and fluorescent molecule following the  
reacting step.

20

19. The fluorescent conjugate prepared according to the  
process of claim 18, wherein the providing step provides a  
heterofunctional linking reagent which is: a heterofunctional  
polyethylene glycol (PEG) derivative, a long chain form of  
25 succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate  
(SMCC), a long chain form of succinimidyl 3-(2-  
pyridyldithio)propionate (SPDP), or, a heterofunctional peptide  
derivative.

30

20. The fluorescent conjugate prepared according to the  
process of claim 19, wherein the providing step provides a  
heterofunctional PEG derivative which comprises N-  
hydroxysuccinidyl (NHS) esters of poly (oxyethylene)  
derivatives containing a maleimide or a vinyl sulfone group or

containing an moiety having essentially the same electrophilicity as the maleimide or the vinyl sulfone group.

21. The fluorescent conjugate prepared according to the process of claim 19, wherein the providing step provides a long chain form of SMCC which comprises succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxy-(6-amido-caproate).

22. The fluorescent conjugate prepared according to the process of claim 19, wherein the providing step provides a long chain form of SPDP which comprises succinimidyl-6-[3-(2-pyridyldithio)propionamido] hexanoate.

23. Use of an antioxidant, a protein, an oxygen depleting system, or a cross linking substance to impair the phototoxic degradation of a biological reagent in a fluorescent conjugate comprising the biological reagent and a fluorescent molecule, or to impair the phototoxic degradation of the material used to prepare a composition comprising a fluorescent molecule, by combining an antioxidant, a protein, an oxygen depleting system, or a cross linking substance with the fluorescent conjugate, or with the material used to prepare a composition comprising a fluorescent molecule.

24. The method in accordance with claim 23 comprising steps of:

providing a material to prepare a composition comprising a fluorescent molecule;

providing an antioxidant, a protein, an oxygen depleting system, or a cross linking substance; and,

combining the material to prepare a fluorescent composition with the provided antioxidant, protein, oxygen depleting system, or cross linking substance, whereby phototoxic degradation of the material used to prepare a

fluorescent composition is impaired upon exposure of that material to a reactive species.

25. The method in accordance with claim 23 comprising  
5 steps of:

providing a fluorescent conjugate;

providing an antioxidant, a protein, an oxygen depleting system, or a cross linking substance; and,

10 combining the fluorescent conjugate with the provided antioxidant, protein, oxygen depleting system, or cross linking substance, whereby phototoxic degradation of the fluorescent conjugate is impaired.

26. A method to facilitate preparation of a stable fluorescent composition, where the composition comprises a  
15 fluorescent molecule which absorbs light and provides a fluorescent result upon exposure to light of a defined spectrum, said method comprising:

20 providing an environment comprising diminished amounts of light from the defined spectrum, relative to a white light environment;

providing the fluorescent molecule, or a material to prepare the composition;

placing the fluorescent molecule or the material to prepare the composition into the provided environment.

25

27. A method in accordance with claim 26, which comprises:

providing the fluorescent molecule;

providing the material to prepare the composition;

30 placing the fluorescent molecule into the provided environment;

placing the material to prepare the composition into the provided environment; and,

35 combining the fluorescent molecule and the material to prepare the composition;

whereby phototoxic degradation of the material to prepare the composition is diminished upon completion of the method.

28. The method of claim 27 wherein the combining step  
5 occurs before the step of placing the fluorescent molecule into the provided environment and before the step of placing the material to prepare the composition into the provided environment.

10 29. The method of claim 26, further comprising a step of associating the material to prepare the composition with the fluorescent molecule, whereby a fluorescent conjugate is produced, and whereby phototoxic degradation of the fluorescent conjugate is diminished.

15 30. A method in accordance with claim 26 to prepare a composition comprising a fluorescent molecule, where the fluorescent molecule absorbs red light and provides a fluorescent result upon exposure to light of a red spectrum,  
20 said method comprising:

providing the fluorescent molecule which absorbs red light and provides a fluorescent result upon exposure to light of a red spectrum;

providing the material to prepare the composition;

25 providing an environment comprising diminished amounts of light from the red light spectrum, compared to a white light environment;

placing the fluorescent molecule into the provided environment comprising diminished amounts of red light;

30 placing the material to prepare the composition into the provided environment comprising diminished amounts of red light; and,

combining the fluorescent molecule and the material to prepare the composition,

whereby phototoxic degradation of the material to prepare the composition is diminished .

5 31. The method of claim 30 wherein the combining step occurs before the step of placing the fluorescent molecule into the provided environment and before the step of placing the material to prepare the composition into the provided environment.

10 32. The method in accordance with claim 30 wherein the environment providing step comprises providing an environment comprising enhanced amounts of green light and diminished amounts of red light.



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US98/06793 <b>(22) International Filing Date:</b> 6 April 1998 (06.04.98) <b>(30) Priority Data:</b> 08/837,309 9 April 1997 (09.04.97) US <b>(71) Applicant:</b> BIOSITE DIAGNOSTICS INCORPORATED [US/US]; Suite D, 11030 Roselle Street, San Diego, CA 92121 (US). <b>(72) Inventors:</b> BEUCHLER, Kenneth, F.; 12523 Manifesto Place, San Diego, CA 92071 (US). MCPHERSON, Paul, H.; 1449 Elva Court, Encinitas, CA 92024 (US). SUNDQUIST, Alfred, R.; 4562 Felton Street, San Diego, CA 92116 (US). <b>(74) Agents:</b> LITHGOW, Timothy, J. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <b>(88) Date of publication of the international search report:</b> 30 December 1998 (30.12.98)	
<b>(54) Title:</b> COMPOSITIONS AND METHODS FOR INHIBITING LIGHT-INDUCED INACTIVATION OF BIOLOGICAL REAGENTS		
<b>(57) Abstract</b> <p>Disclosed is a fluorescent conjugate comprising a biological reagent, a fluorescent molecule, and a means for impeding phototoxic degradation of the biological reagent. The impeding means can be a cross-linking substance having a long molecular distance, whereby the cross-linking substance links the fluorescent molecule and the biological reagent; a quencher of singlet oxygen; a quencher of a free radical; or a combination thereof. Also disclosed is a solution comprising a fluorescent conjugate of a biological reagent and a fluorescent molecule together with an oxygen depleting system.</p>		

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# INTERNATIONAL SEARCH REPORT

Interr. nat. Application No

PCT/US 98/06793

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 G01N33/533 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 442 372 A (HOECHST AKTIENGESELLSCHAFT) 21 August 1991	1, 2, 7, 8, 10, 14-16, 18-20, 23-25
Y	see claims	11-13, 21
X	WO 91 18006 A (DIATRON CORPORATION) 28 November 1991	1, 2, 7, 8, 10, 14-16, 18-20
	see page 15, line 16 - page 16, line 31; claims 1, 23-48	
X	WO 92 18868 A (BIOSITE DIAGNOSTICS INCORPORATED) 29 October 1992	1
Y	see page 12, line 4 - page 13, line 2; claim 5; examples 1, 6	21
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

24 September 1998

Date of mailing of the international search report

01/10/1998

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# INTERNATIONAL SEARCH REPORT

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 08772 A (BIOSITE DIAGNOSTICS INCORPORATED) 30 March 1995 see abstract see page 12, line 36 - page 13, line 6	11-13
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